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EFFECT OF cAMP ON MACROMOLECULE SYNTHESIS IN THE PATHOGENIC PROTOZOA *TRYPANOSOMA CRUZI*

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Macromolecule synthesis of Trypanosoma cruzi *in culture was monitored using radioactive tracers. Cells of different days in culture displayed a preferential incorporation of precursors as follows: 1 day for (^{3}H)-thymidine cells; 3 days for (^{3}H)-uridine cells, and 4 days for (^{3}H)-leucine cells. Autoradiographic studies showed that (^{3}H)-thymidine was incorporated in the DNA of both kinetoplast and nucleus in this order. Shifts in the intracellular content of cAMP either by addition of dibutyryl-cAMP or by stimulation of the adenylcyclase by isoproterenol, caused an inhibition in the synthesis of DNA, RNA and proteins. Addition to the* T. cruzi *cultures of these agents which elevate the intracellular content of cAMP provoked an interruption of cell proliferation as a result of the impairment of macromolecule synthesis. A discrimination was observed among the stereoisomers of isoproterenol, the L configuration showing to be the most active.*

Key words: macromolecule synthesis – radioactive tracers – autoradiographic studies – cAMP – Dibutyryl-cAMP – isoproterenol

Cyclic AMP as a second messenger has been implicated in a variety of biological processes within eucaryotic cells, including growth control (Pastan et al., 1975). From the data found in the literature it is apparent that different types of cells respond in a singular manner to shifts in the intracellular content of cyclic AMP.

Although the influence of cAMP on cell proliferation has been subject of considerable controversy, it has also been shown to act as a mitogenic signal for Swiss 3T3 cells (Rosengurt et al., 1981). The opposite was found for protozoa: In *Leishmania* (Walter, 1981) and *Trypanosoma cruzi* (Oliveira et al., 1984) high levels of cAMP caused inhibition of growth. Also in *Trypanosoma lewisi* (Strickler & Patton, 1975) and *Trypanosoma brucei* (Mancini & Patton, 1981) it was found that cyclic nucleotide has an effect on growth and differentiation.

We have previously reported that high intracellular levels of cAMP caused by stimulation of the adenylcyclase under the action of adrenergic ligand isoproterenol, phosphodiesterase inhibitors, or dibutyryl cyclic AMP, inhibited proliferation of *T. cruzi* (Oliveira et al., 1984).

The aim of the present paper is to further investigate the site of action of cyclic AMP in the chain of molecular events leading to the inhibition of cell division in *T. cruzi*. With this purpose we have investigated the synthesis of macromolecules using radioactive tracers in kinetic experiments during shifts in the intracellular content of cAMP.

MATERIALS AND METHODS

Cell cultures – *Trypanosoma cruzi* epimastigotes, Y strain, obtained from the Department of Protozoology, Instituto Oswaldo Cruz, were grown at 28 °C in LIT medium consisting of 3g bacto-liver infusion broth, 5g bacto-tryptose, 15g yeast extract, 1g NaCl, 0.4g KCl, 1g dextrose, 6.5g Na_2HPO_4, 100ml calf serum, 20ml bovine hemoglobin, and 1500 U/ml of penicillin for 1 liter (Camargo, 1964). Cells were maintained by weekly passages to a new medium and the inocula consisted of stationary phase cells.

Growth experiments – The cells (10^6 cells/ml) were grown in LIT medium with addition of test substances previously sterilized by filtration through Millipore filters. At stationary

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phase (7th day of growth) samples were taken for light microscopy, Giemsa staining and cell counting in a hemocytometer.

Radioactive thymidine, uridine and leucine incorporation – Cells were harvested in various phases of the cell cycle by centrifugation at 1000 x g for 10 min, washed three times with phosphate-buffered saline, and counted in a hemocytometer. The method of Hershko et al. (1971) was used as follows: 10^6 cells/ml were incubated at 28 °C in a buffer solution containing 25 mM Tris-HCl, 100 mM glucose, 123 mM NaCl, 42 mM KCl, 2.6 mM $CaCl_2$, and 2.5 mM $MgSO_4$, pH 7.2, containing one of these radioactive precursors: (methyl-^{3}H)-thymidine (1 uCi/ml), (^{3}H(G))-uridine (0.1 uCi/ml) or (4.5-^{3}H-(N))-L-leucine (0.5 uCi/ml). At the end of the labelling period the cells were chilled in an ice bath, washed three times with cold phosphate-buffered saline and dissolved in 1 ml of 0.01% sodium dodecylsulphate. Following the addition of 0.1 ml 100% (w/v) TCA, the samples were filtered through glass fiber filters (Whatman GF/A).. The filters were washed three times with cold phosphate-buffered saline, dried and counted in a scintillation counter (Beckman model LS-7000).

Autoradiography – Cells were washed three times in KRT buffer (Tris HCl pH 7.2, 25 mM; $MgSO_4$ 1.2 mM; $CaCl_2$ 2.6 mM; KCl 48 mM; NaCl 120 mM and glucose 10 mM), and resuspended in the same buffer plus 10 uCi (^{3}H)-thymidine. Incubation proceeded for 1 hour at 28 °C and then the cells were extensively washed with 1 mM thymidine in KRT buffer. The resulting pellet was resuspended in the same buffer, an aliquot was taken for radioactivity counting, and a samer done over previously prepared slides (1.0g gelatin, 0.05g chrome alum ($K(Cr(So_6H_9)_2\ (H_2O_2).6H_2O$) in 100 ml of distilled water at 65 °C. The slides were dryed at room temperature and treated with a methanol: acetic acid solution (3:1 v/v) for 30 min, and then they were prepared for autoradiography by standard procedures employing Kodak NTB2 emulsion (Kopriwa & Leblond, 1961). After exposure for 3 days, autoradiographs were developed with Kodak D19, fixed in acid fixer and counterstained with Giemsa stain (1:40) for 50 min. Autoradiographs were observed by two investigators using a double-blind method for differential cell counting. For S phase determination the following relation was used:

$$\frac{\text{number of cells with silver grains in the nucleus}}{\text{total number of cells}} \times \text{length of cell cycle}$$

Autoradiographs were photographed with a photomicroscope Zeiss model III with Kodak Panatomic-X film.

Materials – Isoproterenol isomers (L, D and DL); N^4, O^{21}-dibutyryl adenosine 3', 5' cyclic monophosphate (dcAMP); DL-propranolol and ascorbic acid were from Sigma Chemical Company, USA. Thymidine, (methyl-^{3}H) (6.7 uCi/mmol; uridine (^{3}H(G)) 6.0 Ci/mmol and leucine L (4.5-^{3}H(N)) 59 Ci/mmol were purchased from New England Nuclear/ Dupont USA. Cell culture components were from Difco and salts from Merck Darmstadt. Isoproterenol was used in the form of L-isoproterenol, unless mentioned otherwise

RESULTS

T. cruzi epimastigotes cultivated in LIT medium reached stationary phase in the 7th day with a generation time of 24 hours. Although the cells were not completely synchronized, a lag, an exponential, and a stationary phase could be detected in the first, the third and the seventh day of culture respectively (data not shown).

Whenever cells of different stages were incubated with radioactive thymidine, the lag phase cells (24 h) displayed a higher uptake of this precursor than the others (Fig. 1A); it shows that although DNA synthesis is succeeded throughout the other days in culture, in the first 24 hours there was a larger number of cells engaged in DNA synthesis.

It was confirmed by autoradiography that the (^{3}H)-thymidine incorporation into acid-precipitable material was a real indicator of DNA synthesis in these cells, where silver grains could be seen in DNA-containing organelles, namely, nucleus and kinetoplast (Fig. 2A, B). Although most of cells had grains in both organelles, about 20% presented the label only in the kinetoplast (Fig. 2C). On the other hand, silver grains only in the nucleus were rarely seen, thus suggesting that DNA synthesis in the kinetoplast preceeds the one in the nucleus.

Proceeding with the investigation of macromolecules synthesis in *T. cruzi* we have checked

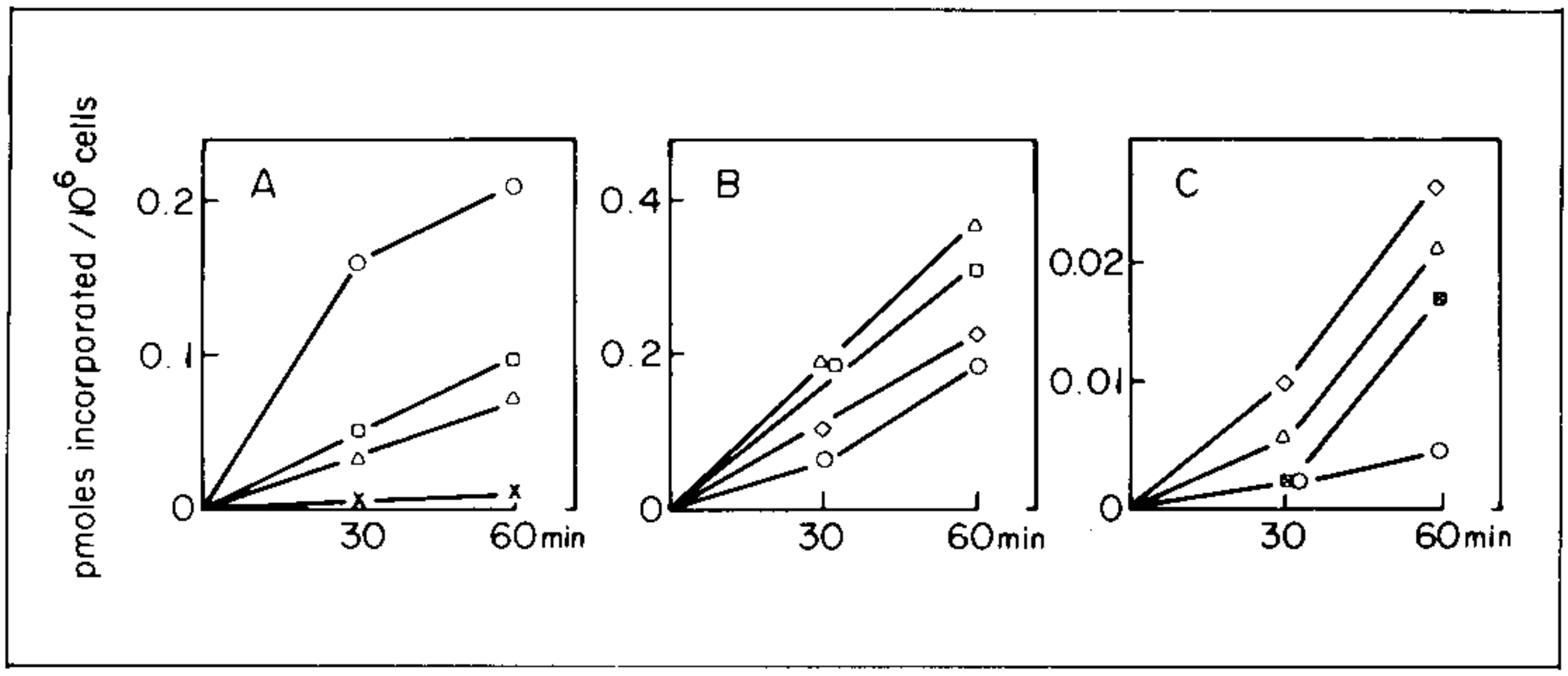

Fig. 1: differential incorporation of macromolecules precursors in *T. cruzi* epimastigotes according to the stage in culture. Cells were collected in different days of culture, washed and incubated with the radioactive precursors, as described in METHODS. A: (^{3}H)-thymidine, B: (^{3}H)-uridine, and C: (^{3}H)-leucine incorporation in acid precipitable material. Cells in culture for: 0 — 0 1 day, □ — □ 2 days, △ — △ 3 days, ◇ — ◇ 4 days, □ — □ 5 days, and x — x 7 days.

RNA synthesis in cells of different times in culture. (^{3}H)-uridine incorporation was linear within 60 min and was higher in 3-days-old cells (Fig. 1B).

For protein synthesis (^{3}H)-leucine incorporation gave a measure of the different metabolic activities of cells at different stages in culture media. In this case, 4 days cells displayed the highest rate of incorporation of the radioactive amino acid, whereas lag phase cells had the lowest values (Fig. 1C). These results were taken in to account for the design of further experiments.

In the following set of experiments (displayed in Fig. 3) we have preincubated the cells for 15 min with agents which raised the intracellular content of cAMP, namely the permeant analog dibutyryl cyclic AMP and the adrenergic ligand isoproterenol. For this we have chosen the cells (at different stages in the growth medium) which displayed the highest synthetic activity towards a particular macromolecule, as already shown in Fig. 1.

The results shown in Fig. 3 indicate that d-cAMP inhibited the incorporation of radioactive precursors for the synthesis of DNA (Fig. 3A), RNA (Fig. 3B) and proteins (Fig. 3C), this last case displaying a higher degree of inhibition. Isoproterenol in the concentration used for this set of experiments (0.1 mM) had the most pronounced inhibitory activity on the incorporation of radioactive precursor into DNA macromolecule.

This inhibitory action of dcAMP and isoproterenol on macromolecules synthesis led to a constraint in *T. cruzi* proliferation, as seen on the Table. The cAMP analog (1 mM) provoked an inhibition of growth to 50% of the control, confirming previous results (Oliveira et al., 1984). Isoproterenol isomers were tested in a concentration of 0.01 mM and in three different experiments, L isoproterenol was the most active isomer, causing a decrease of proliferation in the range of 18 to 33% of the control, whereas D-isomer had very low inhibitory activity or none at all, as in experiment 3 (Table). DL-isoproterenol had intermediate values, as should be expected in a phenomenon mediated by specific receptors, as may be the case of the isoproterenol effect on *T. cruzi*.

DISCUSSION

T. cruzi epimastigotes display different properties according to the stage in culture. It was shown in the present paper changes in the rate of synthesis for DNA, RNA and proteins (Fig. 1). Gonçalves et al. (1980) detected a similar phenomenon when monitoring the levels of the enzyme adenylcyclase and of intracellular cAMP, observing that oscilations on the

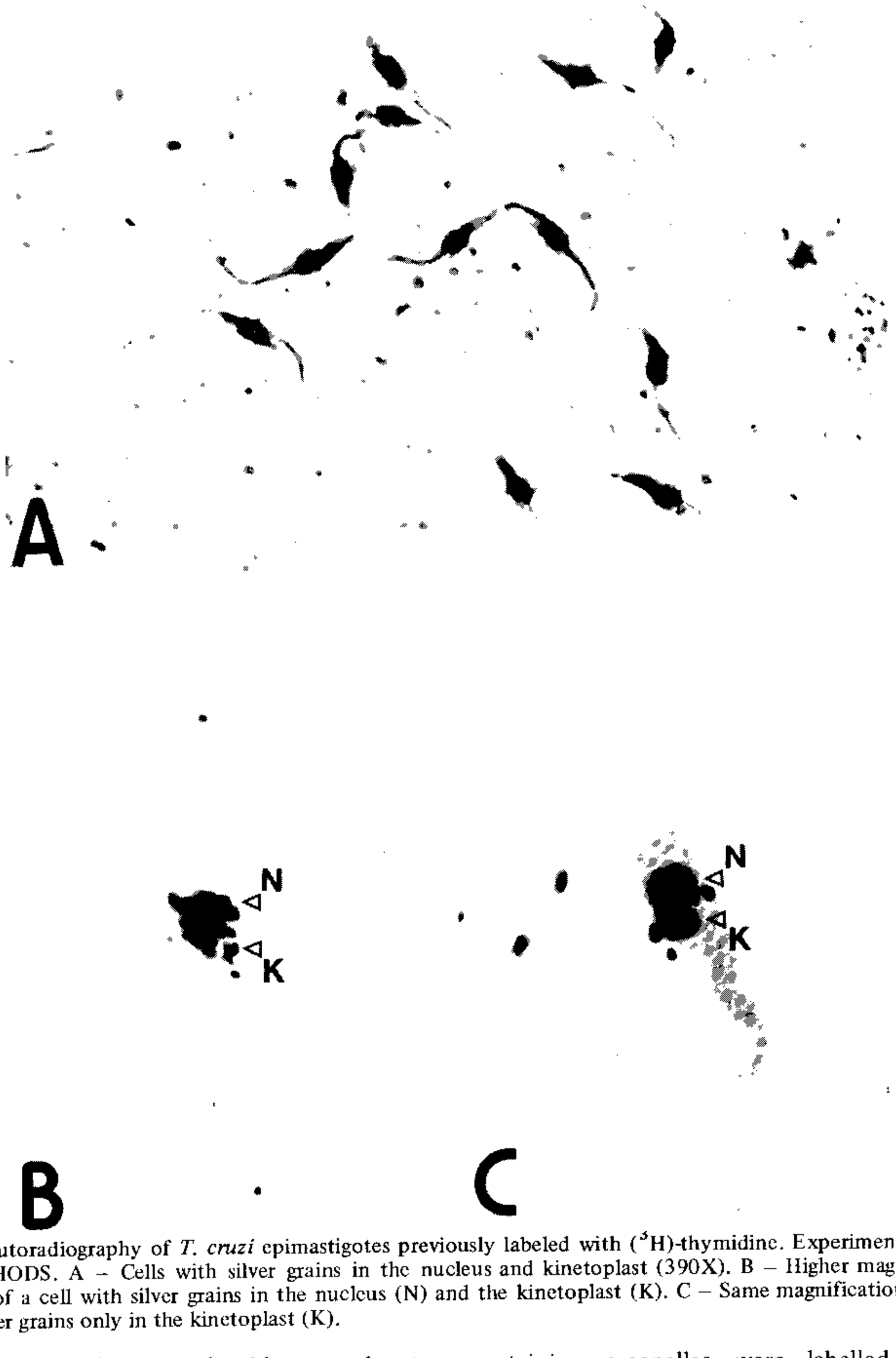

Fig. 2: autoradiography of *T. cruzi* epimastigotes previously labeled with (^{3}H)-thymidine. Experimental details in METHODS. A – Cells with silver grains in the nucleus and kinetoplast (390X). B – Higher magnification (860X) of a cell with silver grains in the nucleus (N) and the kinetoplast (K). C – Same magnification of a cell with silver grains only in the kinetoplast (K).

concentration of this nucleotide were due to alterations on the levels of adenylcyclase, in spite of maintaining the same levels for phosphodiesterase throughout all the stages of culture.

Our experiments with autoradiography enabled us to observe the radioactive thymidine destiny inside the cell. In fact, the DNA-containing organelles were labelled and it seems that the kinetoplast was labelled before the nucleus. The same was observed by Cosgrove & Skeen (1970) in the trypanosomatid *Crithidia fasciculata.* The measurement of the S phase lenght by this method offers obvious limitations (data not shown), but our results were similar with those found by Dvorak (1984) for clone Sylvio-X 10/7 of *T. cruzi* using flow cytometry.

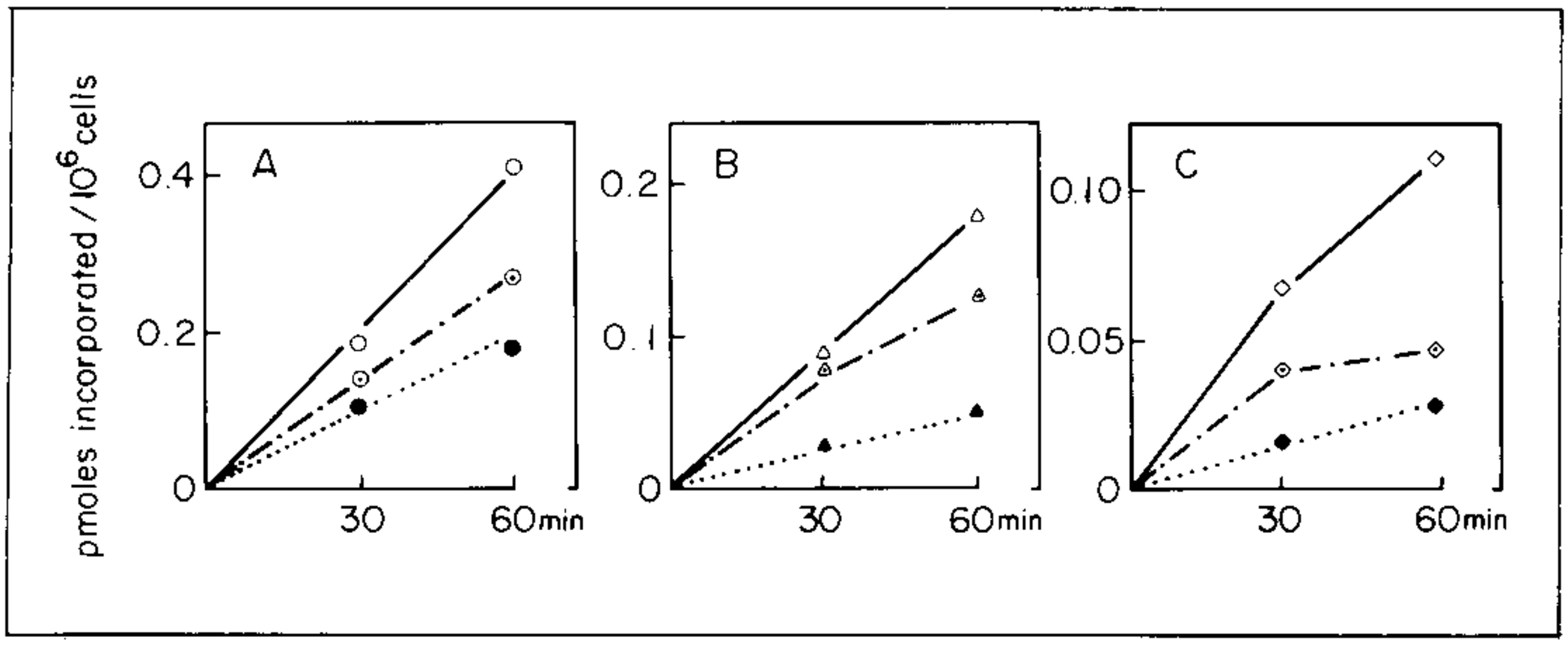

Fig. 3: effect of elevated intracellular levels of cAMP on macromolecules synthesis of *T. cruzi* epimastigotes. Experimental conditions and symbols as in Fig. 1, and details in METHOD. A – (^{3}H)-thymidine, B – (^{3}H)-uridine and C-(^{3}H)-leucine incorporation in acid precipitable material controls: 0 — 0 1 day; △ — △ 3 days; ◇ — ◇ 4 days. Additions to the incubation mixture of either 1 mM dcAMP: ○ – · – ○; △ – · – △; ◇ – · – ◇, or 0.1 mM isoproterenol: ● – – – ●; ▲ – – – ▲; ◆ – – – ◆.

TABLE

Effect of elevated intracellular levels of cAMP on growth of *T. cruzi* epimastigotes*

Exp. No.	No. cells/ml at the start (x10^{-7})	No. cells/ml at stationary phase (x10^{-7})				
		Control	d-cAMP (1 mM)	Isoproterenol (0.01 mM)		
				L	D	DL
1	1.0	11.0 (±0.04)	5.5 (±0.02)	–	–	–
2	0.5	4.4 (±0.02)	–	3.3 (±0.01)	3.9 (±0.06)	4.0 (±0.04)
3	0.5	4.2 (±0.02)	–	2.8 (±0.04)	4.8 (±0.05)	3.9 (±0.02)
4	0.1	3.2 (±0.01)	–	2.6 (±0.09)	3.0 (±0.09)	2.8 (±0.01)

* Values are expressed as the mean ± S. E. M. of duplicates.

In our experiments the synthesis of macromolecules was inhibited when intracellular concentration of cAMP was increased (Fig. 3). A regulatory effect of cAMP has been observed in other biological systems, such as protein synthesis by Rosenfield & Barrieux (1979), or in ribosomal function (Burkhard & Traugh, 1983) as well as DNA synthesis (Willingham et al., 1972). The overall cytostatic effect observed in our experiments (Table) seems to be the addition of an inhibitory action in different steps of the cell biosynthesis.

The results shown on the Table, in which there is a discrimination among the stereoisomers of isoproterenol, are a further confirmation of adrenergic-type receptors present in *T. cruzi* membrane. Wirth & Kierszenbaum (1982) were the first to report a stimulation of adenylcyclase by isoproterenol antagonized by the beta-adrenergic antagonist propranolol in trypomastigotes. Our laboratory has shown the same phenomenon in epimastigotes (insect stage of *T. cruzi*) (Oliveira et al., 1984) and amastigotes (intracellular mammalian stage of *T. cruzi*) (de Castro et al., 1987). The presence of this type of repectors has been determined by the radioligand binding technique (de Castro & Oliveira, 1987).

At present, the role played by the system adrenergic receptors-enzyme adenylcyclase in *T. cruzi* is not fully understood. As an obligatory parasite, this protozoon is in permanent contact with host cell products which, in turn, might act upon the structures on the trypanosome membrane. Further experiments are needed to elucidate this point.

RESUMO

Efeito de cAMP sobre sínteses de macromoléculas no protozoário patogênico *Trypanosoma cruzi* – A síntese de macromoléculas de *T. cruzi* em cultura foi monitorada usando traçadores radioativos. Células de diferentes dias em cultura mostraram uma incorporação preferencial de precursores como se segue: 1 dia para (^{3}H)-timidina; 3 dias para (^{3}H)-uridina e 4 dias para (^{3}H)-leucina. Estudos autoradiográficos mostraram que (^{3}H)-timidina foi incorporada no DNA de ambos, cinetoplasto e núcleo, nesta ordem. Alterações no conteúdo intracelular de cAMP seja por adição de dibutiril-cAMP ou por estimulação de adenilciclase por isoproterenol, causavam inibição na síntese de DNA, RNA e proteínas. A adição destes agentes que elevam o conteúdo intracelular de cAMP em culturas de *T. cruzi* provocou inibição de crescimento, com resultado da síntese macromolecular imperfeita. Foi observada uma discriminação entre os estereoisômeros de isoproterenol, sendo a configuração L, a mais ativa.

Palavras-chave: síntese macromolecular – traçadores radioativos – estudos autoradiográficos – cAMP – Dibutiril cAMP – isoproterenol

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